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13. ABSTRACT ***(Maximum 200 Words)***

uPAR (urokinase-type plasminogen activator receptor) is a key player in metastasis of breast cancer cells. We suggest that uPAR, because it is a GPI-anchored protein, must be present in discrete subregions or "rafts" in the cell surface to function. Our proposal has two parts. First, we will set up systems in our lab for studying signaling through uPAR in cultured human breast cancer cells. Second, we will disrupt rafts, and determine whether signal transduction is affected. Our most important advances this year have been in characterizing lipid rafts, and in determining specific experimental protocols necessary for disrupting them. We have also started to make some headway in characterizing signaling pathways (Yes, MAP kinase) in which rafts are involved. With this foundation laid, the most important focus of the next year will be to improve procedures for examining uPA and uPAR in rafts. It is clear that uPA interactions with uPAR play a key role in metastasis, the deadliest feature of breast cancer. Our findings strongly suggest that the presence of uPAR, a GPI-anchored protein, in membrane rafts affects its signaling and its ability to govern cell migration during metastasis. The need to control this signaling pathway has never been greater.

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INTRODUCTION

uPAR, the urokinase-type plasminogen activator receptor, is a key player in metastasis of breast cancer cells. Our hypothesis is that uPAR must be present in discrete subregions or "rafts" in the cell surface to function. The rationale for this hypothesis is that uPAR is a member of a distinctive class of cell-surface proteins, called GPI-anchored proteins. All these proteins are anchored in the plasma membrane that surrounds each cell by attachment to a lipid molecule, instead of by having a portion of the protein itself pass through the plasma membrane as is true for most proteins on the cell surface. This lipid anchor targets any protein to which it is attached to rafts in the membrane. Work on different members of this class of lipid-anchored proteins has shown that several of them can participate in signal transduction cascades, and in several cases (especially cells of the immune system), these proteins must be present in rafts in order to send signals. Because uPAR is also lipid-anchored, we hypothesize that it must also be in rafts to send signals. Our proposal has two parts. First, we will set up systems in our lab for studying signaling through uPAR in cultured human breast cancer cells. Second, we will disrupt rafts, and determine whether signal transduction is affected. Rafts will be disrupted by three different means, in each case altering the lipid composition of the membrane. All three methods are based on knowledge of how lipids are organized in rafts. We found that lipids in rafts are packed tightly together. Agents that counteract this tight packing disrupt rafts, and may thus inhibit uPAR signaling. In this first year of the award, we have initiated studies to characterize lipid rafts, the association of GPI-anchored proteins with them, and how rafts can be disrupted.

BODY

Task 1. **To establish systems for uPAR signaling in breast cancer cells in out lab, and to determine whether uPAR functions observed in other cells are also seen in breast cancer cells**.

Studies in this Task have been slowed by unexpected difficulties in generating sufficient uPA for these studies. A colleague at Stony Brook, formerly of the Pharmacology Department at Stony Brook, had generated a recombinant baculovirus construct expressing mouse uPA. He had used this material in previous years with good results. He very generously agreed to let us use this construct. Unfortunately, after years of storage, the material was no longer useful. We were unable to obtain purified uPA from this construct. Purified uPA in sufficient amounts for this Task are not available commercially. We are currently proceeding with alternate strategies of obtaining uPA.

Despite this setback, we have performed a number of "feasibility" experiments, to establish systems to be used in the specific experiments outlined in Task 1 once uPA becomes available. These experiments make up the foundation upon which the uPA experiments will rest. This necessary optimization of the systems to be employed will greatly facilitate the uPA experiments, once uPA becomes available. These studies are outlined next.

Raft disruption by cholesterol depletion stimulates the MAP kinase pathway. For convenience, sphingolipid- and cholesterol-rich membrane domains will be referred to here as membrane rafts. As discussed below (Task 2), we have made significant progress in developing useful assays for raft disruption. These techniques will be described there. Here, we report the effect of this disruption on signal transduction pathways culminating in activation of MAP

kinase. A useful model for migration of breast cancer cells during metastasis is to examine the stimulated motility of cultured breast cancer cells in vitro. Previous work has shown that stimulated motility of breast cancer cells involves signaling cascades that culminate in activation of MAP kinase (Nguyen et al., 1998). Our hypothesis, as outlined in Task 1, is that this signaling pathway involves the function of, and is regulated by, lipid rafts. To test this idea, we disrupted rafts by depleting cholesterol. Previous work by us and others has shown that raft integrity and function are completely dependent on membrane cholesterol (Ostermeyer et al., 1999; Sheets et al., 1999). Cyclodextrins are a class of compounds that form hydrophobic pockets in otherwise hydrophilic molecules. Some cyclodextrins bind specifically to cholesterol in this hydrophobic pocket (Kilsdonk et al., 1995). Adding high concentrations of cyclodextrins to cells rapidly and efficiently extracts a high fraction of the total cellular cholesterol (Kilsdonk et al., 1995). As rafts depend on cholesterol, this treatment has turned out to be a very useful tool for disrupting rafts (Ilangumaran and Hoessli, 1998). We used this method to deplete cholesterol from cells grown in tissue culture dishes.

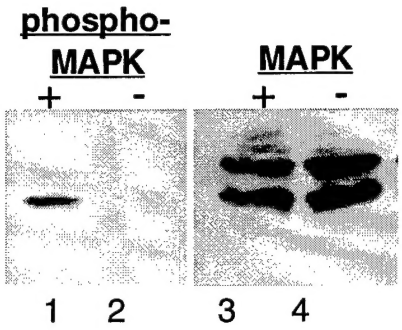

Figure 1. MBCD activates MAP kinase. Cells were (+) or were not (-) treated with MBCD before lysing and analyzing by Western blotting, probing for activated (lanes 1 and 2) or (3 and 4) total MAP kinase.

To examine the effect of cholesterol depletion and raft disruption on signaling though the MAP kinase pathway, we took advantage of the availability of antibodies that specifically recognized activated MAP kinase. MAP kinase becomes phosphorylated when it is activated. Some antibodies recognize only the phosphorylated form. Other control antibodies are also available that recognize MAP kinase in both active and inactive forms. In the experiment shown in Figure 1, we either treated cells with methyl-beta cyclodextrin (MBCD), (marked +) or with vehicle alone (marked -). We then lysed the cells under conditions in which the phosphorylation status of proteins would be maintained. The cell lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS PAGE). Proteins were then transferred to nitrocellulose for Western blotting. The blot was first probed with the activation-specific antibody. As shown in Figure 1, left panel, the activated form of the protein is only seen after MBCD treatment. As a control, to ensure that equal amounts of protein were actually present, the blot was stripped and reprobed with antibodies that recognize the protein regardless of activation state. As shown in Figure 1, right panel, protein levels were very similar in both cases. We conclude, then, that raft disruption greatly stimulates signaling through the MAP kinase pathway. Having established this important fact, and optimized the experimental method, we will be in a very good position to determine whether raft disruption also affects signaling through MAP kinase after stimulation of cells by uPA.

Yes is present and active in membrane rafts, and tyrosine phosphorylates a number of cellular proteins. Non-receptor tyrosine kinases of the Src family have been implicated in signal transduction in membrane rafts in a number of different cell types (Brown and London, 2000). However, it has not yet been established whether these kinase are important in the uPA signaling pathway, and, if so, whether the localization of these kinases to membrane rafts is important in this function. Determining whether this is so is an important goal outlined in Task 1. As a first step in these studies, we have examined the molecular characteristics of a prototype raft-

associated Src-family non-receptor tyrosine kinase, $p62^{Yes}$ (Yes). We have found that active, tyrosine-phosphorylated Yes is present in membrane rafts (Figure 2). In Figure 2A, rafts were incubated with ^{32}P-ATP , and phosphorylated proteins were then detected by SDS-PAGE and autoradiography. Yes and a number of cellular substrates become tyrosine phosphorylated, as demonstrated by the *in vitro* kinase assay shown in Figure 2. Rafts were incubated with unlabelled ATP either on ice, as a control (Panel B, left lane) or at 37° to allow phosphorylation to occur. Proteins were then analyzed by SDS-PAGE and Western blotting, probing with anti-phosphotyrosine antibodies. Figure 2B reveals a number of substrates after the reaction. Only Yes itself is tyrosine phosphorylated before the reaction, as Yes is constitutively phosphorylated on an inhibitory site. Upon activation, it autophosphorylates a separate, activating site. To demonstrate that kinase activity was actually due to Yes, Yes was immunoprecipitated from solubilized rafts, and then subjected to the *in vitro* kinase assay. Immunoprecipitated Yes phosphorylated a number of cellular substrates (not shown). We conclude that Yes is present and active in rafts. When uPA becomes available, we will determine whether it further activates raft-associated Yes. We also note that these experiments demonstrate that Yes phosphorylates a number of substrates. Further work will show whether a subset of these are focal adhesion proteins, as proposed in Task 1. Antibodies to a number of the proteins are commercially available, facilitating this task.

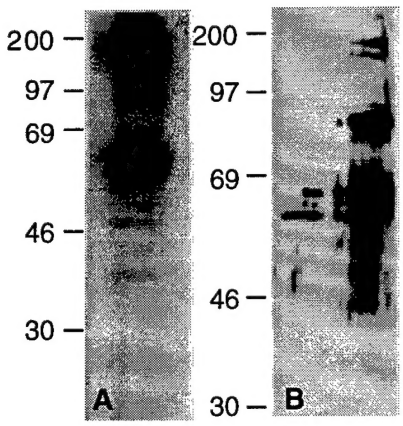

Fig. 2. Yes is active in rafts. A.; Isolated rafts were subject to an in vitro kinase assay including ^{32}P-ATP. Labeled proteins are shown in this autoradiogram. The ca. 60 kDa protein is Yes (not shown). B; Isolated rafts were incubated on ice (1st lane) or at 37° (2nd lane) with cold ATP. Tyrosine phosphorylated proteins are shown in this Western blot.

Task 2. **Disrupt cholesterol and sphingolipid-rich rafts**.

This task was originally proposed for months 13-24 of the award period. However, given the slower-than-expected pace of obtaining purified uPA (outlined above), we have initiated experiments in Task 2 sooner than originally proposed. In fact, most of our progress over the past year has been in Task 2. Our findings will be described next.

MBCD rapidly and efficiently reduces cholesterol levels. As described above, an important finding of our previous work is that rafts in cells require high amounts of membrane cholesterol (Ahmed et al., 1997; Schroeder et al., 1998). This has led to the realization that removing cellular cholesterol can disrupt rafts. This property has been used widely in the field to study a variety of raft-dependent functions. As outline above, one of the most effective ways of depleting cells of cholesterol is to treat them with MBCD. We have performed a careful analysis of the ability of MBCD to remove cholesterol from cultured cells. Cancer cells were plated in normal media and allowed to adhere to the dish. Media was then removed, and then replaced with media lacking serum or other sources of cholesterol, but containing MBCD. Preliminary experiments showed that 10 mM MBCD gave optimal effects (not shown). We also found that

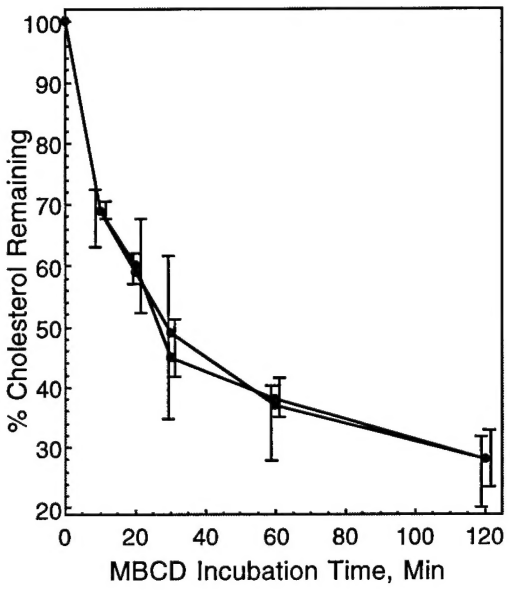

Figure 3. Cholesterol removal by MBCD. Two cancer cell lines were incubated with MBCD for the indicated times. Remaining cholesterol was measured by HP-TLC.

cells remained healthy and adherent for at least 2 hours. After 2 hours in MBCD, cells began to round up and detach from the dish. For this reason, all of our further experiments will be performed at times shorter than 2 hours. At various times after addition of MBCD, individual plates of cells were harvested, after washing several times to remove cholesterol-MBCD complexes. We then extracted lipids from the cells and analyzed them, using a quantitative high-performance thin layer chromatography (HP-TLC) assay that we have used successfully in the past (Brown and Rose, 1992; Melkonian et al., 1995). In this assay, lipids are separated by running in two solvent systems sequentially. The first system separates the more polar lipids, while the second separates the less polar lipids (including cholesterol). A standard curve, consisting of purified lipids in known amounts, is run separately on the same plate. The plate is then sprayed with cupric acetate in a phosphoric acid-containing solution, and heated to char the lipids. Under these conditions, the amount of charring is directly proportional to the amount of lipid. Charring can be measured using a densitometer. It is thus possible to quantitate all the lipids in the cell, using a rapid and sensitive technique. We extracted total cellular lipids from cells treated for various times with MBCD and analyzed them by this method. Importantly, lipids other than cholesterol served as internal controls. We were able to verify that levels of all lipids other than cholesterol remained constant over the course of the assay (not shown). Thus, changes in cholesterol levels were significant, and did not simply reflect cell loss. Results are presented in Figure 3. It is clear that MBCD rapidly and efficiently selectively removes cholesterol.

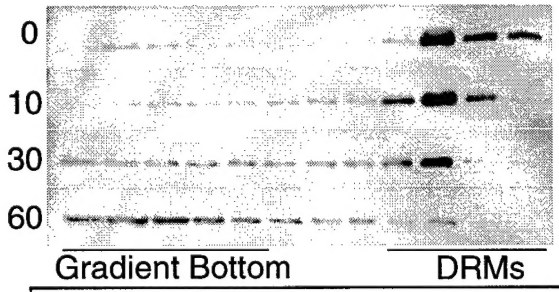

Figure 4. MBCD reduces raft-association of the GPI-anchored protein PLAP. Cancer cells expressing PLAP were incubated for various times (in min) with MBCD before preparation of DRMs by flotation on sucrose gradients. Non-DRM proteins remain at the bottom of the gradient .

MBCD treatment reduces raft-association of the GPI-anchored protein, PLAP. We proposed in Task 2 to measure the partitioning of several molecules between rafts and Triton-soluble fractions after rafts disruption by several means. We now report the effect of raft disruption by MBCD treatment on this association. We first proposed to examine raft-association of uPAR. Experiments in this direction are underway. However, we have experienced unexpected difficulties in antibody recognition of uPAR on blots (not shown). We suspect that our

difficulties with the antibodies we have tried so far stem from cell-type differences in recognition. As a number of different anti-uPAR antibodies are available from several commercial sources, we anticipate that this will be a fairly short-term limitation, and that we will be able to obtain usable antibodies very soon. In the meantime, we have taken advantage of the similar behavior with regard to raft association of uPAR and other GPI-anchored proteins by examining the effect of MBCD treatment on another GPI-anchored protein, placental alkaline phosphatase (PLAP). Cells expressing PLAP were treated with 10 mM MBCD for varying lengths of time, up to 60 min, as indicated in Figure 4. We then extracted the cells with Triton X-100, adjusted the lysates to a high concentration of sucrose, placed them in ultracentrifuge tubes, overlaid them with solutions containing lower concentrations of sucrose to form a step gradient, and centrifuged to equilibrium. This procedure causes DRMs to float to a low-density position high in the gradient (Brown and Rose, 1992). Gradients were fractionated, and samples of each fraction were subjected to SDS-PAGE and Western blotting analysis, probing for PLAP. Results are shown in . In control cells, PLAP is highly concentrated near the top of the gradient, in the DRM-containing fractions. As cholesterol is progressively removed by incubation with MBCD for longer times, it is clear that more PLAP remains in the bottom fractions of the gradient, corresponding to the fully-solubilized non-raft (non-DRM) fractions. By 60 min, at least 50% of the protein is in these non-raft fractions. This results shows that a GPI-anchored protein is displaced from rafts upon cholesterol depletion

MBCD treatment reduces the raft-association of the cholesterol that remains in the cell. MBCD is known to preferentially remove non-raft cholesterol from cells. However, cholesterol is expected to be in equilibrium between raft- and non-raft domains in the cell membrane. Thus, removing cholesterol – even from non-raft domains- is expected to quickly deplete raft cholesterol pools as well, leading to at least partial raft disruption. To test this idea, we developed a method for analyzing the fraction of the cholesterol still in the cell at various times after MBCD addition that was in rafts. To do this, we took advantage of the fact that cells can be labeled with ^{3}H cholesterol. As this cholesterol cannot be degraded or metabolized by cells (except to cholesteryl ester, expected to occur for only a small fraction of total cholesterol), the ^{3}H cholesterol that is added will equilibrate among all the cholesterol-containing membranes in the cell. It will thus act as a good marker for total cellular cholesterol. Cells were labeled to steady-state with ^{3}H cholesterol in this manner. They were then treated with MBCD for varying times, extracted with Triton X-100, and subjected to sucrose density gradient centrifugation to equilibrium as described above to separate raft and non-raft fractions. Gradients were fractionated, and radioactivity was measured. Data are presented in Table I. By summing all the counts in the gradient, and comparing with the untreated control, we were able to determine the fraction of total ^{3}H-cholesterol that remained in the cells at each time. (This agreed well with the amounts determined by HP-TLC.) Furthermore, by summing counts in raft and non-raft fractions

Time in CD, min	% of Initial [^{3}H]Chol Remaining	% of [^{3}H]Chol in DRMs
0	100	58 ± 3
10	63 ± 15	44 ± 7
30	46 ± 11	40 ± 6
60	32 ± 2	31 ± 6

Table I. Cells prelabeled with [^{3}H]cholesterol were treated with MBCD for the indicated times and then extracted with Triton X-100. Lysates were subjected to sucrose gradient ultracentrifugation,

respectively, we were able to determine the fraction of the total ^{3}H-cholesterol remaining in the cells at any time that was present in rafts. This clearly showed that the fraction of cholesterol present in rafts decreased with increasing time in MBCD.

Together, results shown in Figure 4 and Table I show that treating cells with MBCD causes a progressive release of both a GPI-anchored proteins and cholesterol from rafts. We will now be in a very good position to determine whether uPAR and other components of its signaling pathway are similarly affected.

Sphingomyelinase treatment efficiently reduces sphingomyelin levels. We have shown that rafts require both cholesterol and sphingolipids (Ahmed et al., 1997; Schroeder et al., 1998). Thus, an alternate method of disrupting rafts is to deplete cells of sphingolipids. In Task 2, we propose to do this using beta-chloroalanine. This is an inhibitor or sphingolipid synthesis. Cells are treated with this compound for several days. Over this time, existing sphingolipids are gradually turned over, or diluted out as cells grow and divide. Cells become progressively depleted of sphingolipids. We have initiated studies with beta-chloroalanine. However, we have concentrated harder on an alternate path toward the same goal. The enzyme sphingomyelinase rapidly and efficiently cleaves the head group from sphingomyelin, destroying it. The advantage of this method over beta chloroalanine is its speed. Cells are rapidly depleted of sphingomyelin, allowing examination of acute effects of this treatment. With metabolic depletion, as occurs in the case of beta-chloroalanine, cells may induce compensatory mechanisms over several days, making it more difficult to discern the actual effects of the treatment. There are two major classes of sphingolipids in mammalian cells: sphingomyelin and glycosphingolipids. Sphingomyelinase, as mentioned above, rapidly destroys sphingomyelin. However, there are no comparable methods for destroying glycosphingolipids. Thus, glycosphingolipids remain after sphingomyelinase treatment, complicating interpretation of the results. For this reason, we did not propose to disrupt rafts using sphingomyelinase in the original proposal. Since then, however, we have obtained a very useful line of cancer cells that cannot make glycosphingolipids. These were developed by Hirabayashi and colleagues (Ichikawa et al., 1994). Although these are melanoma cells and not breast cancer cells, we believe that principles derived from melanoma cells will apply to breast cancer cells as well. For this reason, we have treated these cells with sphingomyelinase. Cells were treated for 30 minutes with 5 units/ml sphingomyelinase (or were treated with vehicle alone as a control). This short time assured that accumulation of signaling compounds generated from sphingomyelin breakdown didn't affect the results. We then extracted lipids and subjected them to quantitative HP-TLC as described above. Figure 5 shows the amount of sphingomyelin in treated and control cells, visualized by charring. Quantitation of the results by comparison to standards revealed that 80% of the total cellular sphingomyelin was degraded by this method.

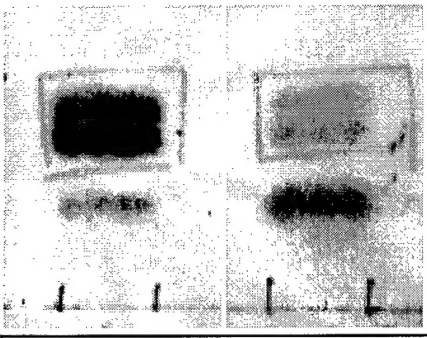

Figure 5. Sphingomyelinase removes most sphingomyelin. Cells were treated (right) or not (left) with sphingomyelinase. Lipids were extracted and analyzed by HP-TLC (origin marked with a line), visualized by charring. The sphingomyelin doublet is boxed.

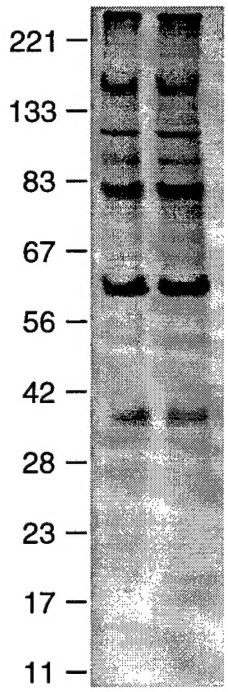

Figure 6. Effect of sphingomyelinase treatment on DRM protein profile. ^{35}S-methionine-labeled cells were treated (right lane) or not (left lane) with sphingomyelinase before DRMs were prepared and DRM proteins analyzed by SDS-PAGE and autoradiography.

Sphingolipid depletion does not fully disrupt rafts. As the cells described above lack glycosphingolipids, sphingomyelinase treatment reduces total sphingolipid levels by 80%. This is about the same as the level of cholesterol depletion by MBCD. As described above, this level of cholesterol disruption significantly affects the properties of rafts. However, we obtained an unexpected result when we examined sphingolipid depletion. We first used our standard detergent insolubility assay for raft recovery. Lysates were then adjusted to a high density of sucrose and placed in an ultracentrifuge tube. A sucrose gradient was layered over the lysate, and was spun to equilibrium. Detergent-insoluble rafts were recovered from the interface between medium-density and low-density steps in the gradient (Melkonian et al., 1995). Surprisingly, we visually observed good recovery of detergent-resistant membrane (DRMs) rafts, even after sphingomyelinase treatment (not shown). As this is a non-quantitative measure of DRM yield, we also examined the protein profile of these DRMs, as a way of determining both quantitatively whether protein yield in DRMs was reduced by sphingomyelinase treatment, and whether different proteins were extracted to different extents before and after treatment. Cells containing proteins labeled to steady-state with ^{35}S-methionine, with (Figure 6, right) or without (Figure 6, left) sphingomyelinase treatment, were lysed in cold Triton X-100, as we have described earlier (Brown and London, 1998). Results (shown in Figure 6) revealed that very similar DRM protein profiles were obtained in each case. Thus, in contrast to cholesterol depletion, cleavage of sphingomyelin in glycosphingolipid-negative cells had little detectable effect on the ability of proteins to associate with DRMs. We speculate that this may result from the ability of ceramide, which is generated from sphingomyelin after cleavage, to support raft formation. For this reason, we will now return to the use of beta-chloroalanine to deplete sphingolipids, as this compound inhibits synthesis at a very early stage and is not expected to cause any raft-promoting intermediates to accumulate. However, because of the concerns about beta-chloroalanine outlined above, and because raft disruption by cholesterol depletion is so efficient, we will concentrate primarily on this method in further studies.

Task 3. **Apply the raft-disruption methods in Task 2 to the uPAR functional assays in Task 1**.

Experiments in Task 3, proposed to be completed during months 25-36 of the award period, have not been started yet.

KEY RESEARCH ACCOMPLISHMENTS

As detailed in the Body, the key research accomplishments resulting from this year's work are the following findings:

- Raft disruption by cholesterol depletion stimulates the MAP kinase pathway.
- Yes is present and active in membrane rafts, and tyrosine phosphorylates a number of cellular proteins.
- MBCD rapidly and efficiently reduces cholesterol levels.
- MBCD treatment reduces raft-association of the GPI-anchored protein, PLAP.
- MBCD treatment reduces the raft-association of the cholesterol that remains in the cell.
- Sphingomyelinase treatment efficiently reduces sphingomyelin levels.
- Sphingolipid depletion does not fully disrupt rafts.

REPORTABLE OUTCOMES

The principle investigator gave presentations including work described here at the following venues:

6/12/00; Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown, MA
7/27-7/29 2000; Japanese Society of Carbohydrate Research Annual meeting, Nagoya, Japan
8/6-8/11 2000; FASEB (Federation of American Societies for Experimental biology) summer conference on "Lipid-Modifications of Proteins", Copper Mountain, CO
10/23-24/00; Department of Biochemistry, University of Alberta, Edmonton, Canada
10/30/00; Biology Department, University of Vermont, Burlington, VT
2/18/01; Biophysical Society Annual meeting, Boston, MA
3/21/01; Department of Physiology, Indiana University, Indianapolis, IN
4/6/01; Graduate Seminar Program, Southern Methodist University, Dallas, TX

There were no publications or other reportable outcomes of this work in the past year.

CONCLUSIONS

As detailed in the Body of this report, our most important advances have been in characterizing lipid rafts, and in determining specific experimental protocols necessary for disrupting them. We have also started to make some headway in characterizing signaling pathways (Yes, MAP kinase) in which rafts are involved. With this foundation laid, the most important focus of the next year will be to improve procedures for examining uPA and uPAR in rafts.

As far as the "so what" question goes, the medical significance of this work remains as described in the original proposal. It is clear that uPA interactions with uPAR play a key role in metastasis, the deadliest feature of breast cancer. Our findings strongly suggest that the presence of uPAR, a GPI-anchored protein, in membrane rafts affects its signaling and its ability to govern cell migration during metastasis. The need to control this signaling pathway has never been greater.

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